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13. ABSTRACT (Maximum 200) This ongoing case-control study is being conducted at the Columbia Presbyterian Medical Center (CPMC) and is investigating whether exposures to polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HA), widespread environmental and dietary contaminants are associated with risk of breast cancer. The study is designed to enroll 100 cases and 100 benign breast disease (BBD) controls from whom blood, biopsy tissue and questionnaire data are being collected and 100 healthy controls from whom blood and questionnaire data are being collected. The study is utilizing biomarkers (PAH-, HA-, and smoking related-DNA adducts) as measures of exposure and p53 mutations as a biomarker of pre-clinical effects. Years one and two have focused on patient recruitment, interviewing and laboratory analyses. Progress in patient recruitment has been excellent and is running ahead of schedule with 311 patients currently enrolled. Samples have been analyzed for carcinogen-DNA adduct levels and p53 expression and descriptive statistical analyses of the data are presented. Additionally, samples have been used for several pilot studies and the descriptive analyses for these biomarkers are presented as well.				
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FOREWORD

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INTRODUCTION

Breast cancer afflicts one in nine women by the age of 85 and is the second leading cause of cancer death among American women. The incidence has been increasing steadily in the United States to reach 182,000 new cases in 1993. Known risk factors account for only approximately a third of cases. It is likely that environmental factors (including exposures related to lifestyle, occupation and ambient pollution) are contributors, particularly in high risk areas such as the northeastern United States. Environmental contaminants such as the polycyclic aromatic hydrocarbons (PAH), heterocyclic amines (HA), cigarette smoke constituents and organochlorine residues are suspected mammary carcinogens of concern (1,2,3).

Traditionally, environmental cancer epidemiology has been hampered by difficulties in obtaining accurate data on individual exposures and on individual variation in response to carcinogens. The development of biomarkers has provided a tool that can circumvent these problems by providing individual measurements of the biologic dose of carcinogens, preclinical effects and susceptibility to cancer.

The goals of this project are to determine: 1) whether specific environmental exposures are associated with PAH-, HA-, and smoking related-DNA adducts in mononuclear white blood cells and breast tissue; 2) whether these biomarkers are associated with breast cancer case-control status; 3) whether increased carcinogen-DNA adduct levels are associated with the presence of mutations in the p53 tumor suppressor gene in breast tumors; and 4) to store samples for the piloting of other biomarkers related to potential environmental and susceptibility factors in breast cancer.

1. Environmental Exposures of Interest

Polycyclic aromatic hydrocarbons (PAH) and aromatic amines are the two main classes of mutagenic chemical carcinogens that have consistently induced mammary tumors in experimental bioassays, and there is evidence that these compounds may play a role in human breast cancer development (3,4,1). PAH are ubiquitous pollutants found in ambient air as well as the workplace environment, drinking water and food (5). Incomplete combustion of organic material, including fossil fuels, is the major source of PAH, such as benzo(a)pyrene (BP), which is used as a representative indicator of total PAH concentrations (6).

Human exposure to heterocyclic amines comes principally through the diet. Creatine, amino acids, and sugars derived from muscle are important precursors for production of these mutagens (7). Muscle from meat, chicken, and fish produce similar mutagenic heterocyclic compounds, with temperature and time being the more important determinants of their formation during cooking (8).

Most studies of active smoking have found either a small positive association (about 20-30%) or no association with breast cancer (9,10,11,12,13,14,15). However, few studies have considered age of onset of smoking. A recent study has, in fact, shown that heavy smoking at an early age (before 16) is associated with a greater risk of breast cancer (odds ratio of 1.7) (9). There have been three reports of an increased risk of breast cancer from passive smoking (16). These results require confirmation (15,16). There is compelling evidence that constituents of cigarette smoke reach the breast and damage DNA through adduct formation (see Preliminary Studies) (1,17,18,19).

2. Biomarkers Under Investigation: PAH-, HA-, Smoking related-DNA Adducts and p53 Mutational Spectra

Biomarkers can be used to supplement questionnaire and monitoring data. Extensive data indicate that most carcinogens, including PAH, HA and cigarette smoke constituents, are metabolically activated to electrophilic species capable of covalently binding to cellular macromolecules. In laboratory animals, the carcinogenic potency of a series of genotoxic carcinogens, including PAH, is generally correlated with their ability to form covalent adducts with DNA (20,21).

Therefore, carcinogen-DNA binding is widely viewed as a necessary (though not sufficient) event in cancer induction. Adduct measurements can provide sensitive integrating dosimeters for potential mammary carcinogens. DNA adducts can be quantitated by the ^{32}P -postlabeling method which measures a broad spectrum of adducts on DNA (22). PAH-DNA adducts can also be analyzed by immunologic methods either by an Enzyme Linked Immunosorbent Assay (ELISA) or by immunohistochemistry (23,24,25). Here at Columbia, both methods utilize sensitive antibodies, developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, that recognize PAH-DNA adducts. These techniques can provide a measure of the amount of genotoxic carcinogen that is impinging on the target tissue, often referred to as the biologically effective dose, and can be used as an exposure index in epidemiologic investigations. In the present study PAH-, HA-, and smoking related-DNA adducts are being analyzed in mononuclear white blood cells from cases, benign breast disease (BBD) controls, and healthy controls and in breast tissue from cases and BBD controls.

It has been suggested that mutational spectra in suitable reporter genes, such as p53, can reflect exposures to carcinogens that are strongly implicated in carcinogenesis (26,27,28). The spectrum of mutations found in these reporter genes can be conceptualized as the "fingerprint" left by mutagens that are likely to have contributed to the development of the cancer (29,27,28). p53 is a tumor suppressor gene, the inactivation of which appears to play a critical role in carcinogenesis. In sporadic breast cancer, mutated p53 has been found in approximately 50% of tumors (28,27). p53 is thus a relevant reporter gene in which to analyze the effects of PAH, HAs and cigarette smoke constituents on breast tissue.

Studies of the mutational spectra in breast cancer tumors have shown an increase in G→T transversions in CpG dinucleotides on the non-transcribed strand (27,28,30). G→T transversions appear to occur early in tumor development, and have been detected in all stages of disease with a prevalence of approximately 20% of all mutations (28,31,32). A similar mutational spectrum has been found in lung tumors for which environmental causes are well known (27,33). Combined with the fact that constituents of cigarette smoke (including PAH) are known to cause G→T transversions, this knowledge has led to the suggestion that environmental factors may be responsible for the mutational spectra found in breast cancer (27,28). In addition, HA are also known to induce G→T transversions (30). A finding of an association between PAH-, HA-, or cigarette smoke constituent-DNA adducts and p53 mutations in breast tissue would provide biologically meaningful evidence that these contaminants play a role in breast cancer development.

Other promising biomarkers include the oncogenes, erbB-2, ras p21 and cyclin D1, which are often overexpressed in breast tumors, and blood levels of the DDT metabolite (DDE) which has been associated with breast cancer development in some studies.

3. Preliminary Studies

In a pilot study by Drs. Perera and Phillips, DNA adducts were detected in breast tissue samples by the ^{32}P -postlabeling method using the P1 nuclease extraction procedure (1). This method detects aromatic adducts including those formed by BP and other PAH. Results were available from 31 specimens, including tumor and/or tumor adjacent tissue from 15 women with breast cancer and 5 healthy women undergoing reduction mammoplasty. Among cases, adduct levels ranged from 1.58 to 10.00 adducts/ 10^8 nucleotides, with a mean of 4.69 adducts/ 10^8 nucleotides in tumor tissue, 6.13 adducts/ 10^8 nucleotides in tumor adjacent tissue and 5.3 adducts/ 10^8 nucleotides in tumor and tumor-adjacent tissue combined. These values were in the lower end of the range seen in lung tissue of smokers and nonsmokers. Among "controls" adduct levels ranged from 0.43 to 4.41 adducts/ 10^8 nucleotides with a mean of 2.04 adducts/ 10^8 nucleotides. Smoking histories were available on the 15 cases. DNA samples from 5 of the 10 smokers (tumor and/or tumor adjacent tissue) displayed the

characteristic pattern of smoking-related adducts (a diagonal zone of radioactivity) that has been reported in prior studies of lung cancer patients (34). None of the samples from the 5 nonsmokers showed this characteristic smoking-related pattern. This preliminary data indicated that PAH reach breast tissue and cause genetic damage, and that the measurement of carcinogen-DNA adducts in breast tissue is a useful tool for the epidemiologic study of breast cancer development. These findings have subsequently been confirmed by Li and colleagues (17).

4. Study Design

The current case-control study is designed to include 100 breast cancer cases, 100 BBD controls, and 100 healthy controls. Cases and BBD controls are being recruited from the private practices of Drs. Estabrook and Schnabel at Columbia-Presbyterian Medical Center (CPMC). Healthy controls are currently being recruited from the private GYN practices of Drs. Kelley and Levine at CPMC.

Controls are being frequency-matched to cases on age and ethnic group (African American, Caucasian, Latina). Patients with conditions that are suspected of influencing blood biomarker levels independent of carcinogenesis are being excluded. Exclusion criteria include: prior history of cancer at any site, current pregnancy, breast feeding within the prior three months, and bone fractures within the last six months. Within the BBD study group, patients with diagnoses of benign disease with atypia are being excluded. These diagnoses are associated with an increased future risk for breast cancer and these patients may share common risk factors with the cases.

Blood samples, questionnaire data and pathology reports are being collected from all of the patients, and breast tissue samples are being collected from cases and BBD controls. Blood samples are being fractionated, processed and preserved for the assays to be conducted under this grant and to create a bank of specimens to support future research projects. Under this grant, mononuclear white blood cell (MWBC) samples are being analyzed for PAH-, HA-, and smoking related-DNA adducts and breast tissue samples are being analyzed for PAH-DNA adducts. Additionally, breast tissue samples are being analyzed for mutations in the p53 tumor suppressor gene using immunohistochemistry and mutation detection with gene chip technology.

Statistical analyses will be used to test our major hypotheses. Logistic regression analysis will be used to determine if carcinogen-DNA adduct levels measured in tissue and/or MWBC are associated with case-control status after controlling for confounding variables. Additionally, logistic regression will be used to test the hypothesis that among cases, mutations within the p53 tumor suppressor gene are associated with increased carcinogen-DNA adduct levels in tissue and/or MWBC. Finally, using questionnaire data on environmental, occupational and dietary exposures, associations between life-style factors and carcinogen-DNA adduct formation in MWBC and breast tissue will be investigated.

BODY OF THE REPORT: PROGRESS DURING YEAR TWO

1. Patient Recruitment, Sample and Data Collection

a. Patient Enrollment

Year two of the research project has been completed. Active patient surveillance programs have been conducted in the offices of collaborating breast surgeons, Drs. Estabrook and Schnabel. Cases and BBD controls have been enrolled by two interviewers under the direction of these two surgeons and their staff. All patients undergoing breast surgery with these doctors were evaluated as potential subjects. Eligible patients were identified and enrolled after the physician recommended surgery, but before surgery was performed. The study objectives and the patient's role in the study were explained to each of the prospective subjects and interested patients signed a consent form that

met DOD and CPMC institutional requirements. Following enrollment, the patient was interviewed and a blood sample was drawn. Blood samples were drawn prior to surgery to prevent confounding of biomarker data by exposures to anesthesia, chemotherapy, hormone therapy, biologic changes associated with the healing process, or post-surgical changes in diet. These procedures are continuing.

The most problematic aspect of any case-control study is the selection of an appropriate control group. For this study an appropriate healthy control patient population was defined as women who, were they to develop a breast-related complaint, would likely be referred to Drs. Estabrook or Schnabel. Using this criteria, patients from the private GYN practices of Drs. Levine and Kelley were selected as prospective healthy controls. Their offices are in the same building as the CPMC Breast Service and these doctors refer their patients to Drs. Estabrook and Schnabel for breast health care. Further, data on birth date and residential zip code were analyzed from a random sample of each physician's patients and were found to be similar across the physician's practices. Healthy control subjects are being enrolled under the direction of Drs. Kelley and Levine and their staff. Women are approached during routine GYN check ups and enrolled into the study. These women sign a consent form, donate blood samples and take part in the structured interview.

Additionally, in year two we continued our surveillance and enrollment system in the Vanderbilt Breast and GYN clinics. These clinics serve the Washington Heights, Northern Harlem, and Southern Bronx communities. These communities are largely African American and Latino. Recruitment was conducted with oversight by Drs. Krementz and Claire, directors of the two clinics. Every patient referred to surgery from the breast clinic was evaluated and those meeting the enrollment criteria were invited to join the study. Healthy controls enrolled from the GYN clinics were frequency matched on age and ethnic group to the clinic cases. Our program in these clinics has allowed us to enroll African American and Latina subjects of lower socio-economic status. This is a medically under-served population that has not been included in past studies of breast cancer.

As a result of these surveillance programs, patient enrollment has occurred at a faster pace than originally anticipated (see Table 1). This enhances the strength and validity of study in two ways. First of all, since we have a wide catchment system and are able to evaluate nearly every patient who enters it, we are better able to assure that we have a representative sample of cases. This over-sampling will also assure that we will have complete data and samples (questionnaire and medical record data and tissue and blood samples) for 300 patients. The additional samples collected through these recruitment efforts will be stored for research projects to be conducted under separate funding.

Table 1
Patient Enrollment

Category	Patients
Total Enrolled Patients	311
Cases	87
BBD Controls	81
Healthy Controls	92
Other*	37
Subjects with as yet incomplete Path. reports	14

* includes: benign breast disease with atypia, lobular carcinoma in situ, and rare cancers (eg. Cystosarcoma phylloides).

b. Questionnaire and Pathology Data

Each of the patients took part in a structured interview that covers demographic variables, reproductive and health histories, diet, residential history, smoking, alcohol consumption, occupational history and environmental exposures. Data from the questionnaires are being abstracted into a computer database in an ongoing fashion. Pathology reports and data on receptor, proliferative and clinical markers analyzed by the pathology department (estrogen/progesterone receptor status, erbB-2, DNA index, G0-G1, S, and G2-M cell cycle status) are also being collected and abstracted. Additionally, information on stage and tumor size are being collected from the CPMC Tumor Registry.

c. Biological Specimen Collection and Storage

Blood samples continue to be collected from subjects and separated into total white blood cell, red blood cell, mononuclear white blood cell, and plasma components. In addition to preserving the blood samples for the assays funded under this proposal, our design called for storing of aliquots for future research. Sample aliquots have been processed and stored in anticipation of future analyses of; 1) organochlorines, 2) plasma vitamin C and E, retinoids and carotinoids, 3) hemoglobin adducts, 4) plasma erbB-2 extra-cellular domain, 5) plasma ras levels, 6) plasma p53 levels, 7) plasma EGFR levels, 8) biomarkers of oxidative damage, and 9) metabolic genotype (NAT2, GSTm1, CYP1A1). This has created a sample bank that will allow future research to be conducted in an efficient and economical manner. Under separate funding preliminary analyses of several of these markers have been conducted to support spin-off studies (see **Pilot Studies** and **Complementary Studies**).

Due to the small size of many of the lesions, frozen tissue is not available from all of our patients. Samples of DNA (50 ug) from the larger frozen samples are being collected. Paraffin embedded biopsy specimens from cases and BBD controls are being retrieved from the CPMC Pathology Department. Samples from these specimen blocks are being shaved off with a microtome and stored on glass slides (10-20 slides for immunohistochemical analysis) or in plastic vials (for future DNA extraction). One slide from each patient is being hematoxylin and eosin (H&E) stained to provide a histological reference.

This archive of paired blood and tissue samples and associated questionnaire data and pathology reports is an invaluable resource that supports our current research and will form the basis

of future projects that can be conducted in a timely and efficient manner.

2. Laboratory Component

The analyses of biological samples for carcinogen-DNA adducts and p53 mutations are ongoing; descriptive analyses for these and other markers are presented below and in Tables 2 and 3. Interim analyses of biomarker data with respect to case-control status has not been performed since multiple analyses were not factored into the study design and thus interim analyses will increase the Type I error rate. Further, multiple comparisons may bias the analytical process if Type I errors are made during the interim analyses and undue attention to these variables influences later analytical decisions. To minimize the possibility of a Type I error we have not decoded the samples and performed case-control analyses for the funded biomarkers. As described in our study design case-control analyses will be performed at the end of the study when the datasets have been completed.

a. Postlabelling Analysis of MWBC

Mononuclear white blood cell DNA have been analyzed for the presence of PAH-, smoking related-, and heterocyclic amine-DNA adducts by ^{32}P postlabelling methods in Dr. Phillips' lab. Descriptive data are presented here on the first 75 samples analyzed for PAH-DNA adducts using the P1 nuclease digestion method. The mean adduct level is 6.99 adducts per 10^8 nucleotides with a standard deviation of 3.15 adducts per 10^8 nucleotides. The data showed a log normal distribution which is consistent with our prior studies of PAH-DNA adducts. Each of the postlabelling chromatographs were analyzed for a diagonal radioactive zone (DRZ) which indicates the presence of smoking related-DNA adducts. Chromatographs from 10 of the samples had smoking related-DNA adducts, which is consistent with the low prevalence of current smokers in the study population. These 75 samples were also analyzed for HA using the ATP-deficient postlabelling method. None of the samples exhibited detectable levels of heterocyclic amines.

b. PAH-DNA Adduct Analysis in Breast Tissue

We have used an immunohistochemistry assay to analyze the paraffin embedded tissue samples for PAH-DNA adducts. The assay utilizes a sensitive monoclonal antibody, developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, that is highly sensitive and specific for PAH-DNA adducts. Stained slides are analyzed on a Becton Dickson Cell Analysis System (CAS 200) which measures the Optical Density (OD) of the staining on the slides. The OD results provide a quantitative measure of the amount of antibody staining and thus PAH-DNA adduct levels. Two of the major strengths of this assay are that it works well on paraffin embedded tissue sections and that it only requires very small amounts of tissue. Descriptive data are presented for the initial set of 42 samples from 33 patients. The mean OD of the samples was 0.44 with a standard deviation of 0.11.

c. p53 Analysis

Immunohistochemical techniques have been used to initially screen for p53 mutations. These techniques have been found to correlate well with SSCP techniques for mutant detection (35). p53 analysis by immunohistochemistry is ongoing as tissue blocks are released to us from the Pathology Department. Descriptive data for the initial set of samples analyzed (62 tissue samples from 48 individuals; 24 benign, 23 tumor, and 15 tumor adjacent). Twenty two of the 62 samples were positive by immunohistochemistry which, considering the mix of tissues analyzed, is consistent with published literature (28,27). Samples that are positive by immunohistochemistry are being analyzed for mutational spectra by Affymetrix Inc.'s (Santa Clara, California) gene chip system (36,37,38). In related work we have analyzed 33 samples from 20 individuals from a parallel study of patients from Long Island and we will compare results on p53 in the two populations.

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d. Pilot Studies

One of the aims of this study was to create a sample bank that would support future studies. Under separate funding portions of these samples have been used in pilot studies to support current and future grant proposals for new studies using this sample bank. Data from these pilot studies have been analyzed considering case-control status so that results may be presented in grant applications and to provide a basis for power calculations.

i. Cyclin D1

We are developing a separate proposal to determine whether cyclin D1 can be detected in blood samples from women whose tumors overexpress cyclin D1. The goal of this proposed project is to investigate whether analysis of cyclin D1 in blood samples can be used as a marker of a woman's breast cancer status. In order to identify women whose blood samples were likely to have increased levels of cyclin D1, we have analyzed 41 tissue samples (15 benign, 11 normal adjacent, 15 tumor) from 32 of the present breast cancer case-control study subjects for cyclin D1 protein overexpression. Eleven of the 41 tissue samples were found to strongly overexpress cyclin D1, which is consistent with the published literature (39,40,41). Blood samples from the 7 case patients whose tumor samples were found to strongly overexpress cyclin D1 will be used to develop an assay to detect cyclin D1 in blood.

ii. ras p21 Levels in Blood Samples

In a related project we used an existing technique for detecting ras p21 protein in blood samples to assay stored samples. It has been shown in other cancers that ras p21 can be detected in blood samples from patients who have tumors that have mutated or overexpressed ras (42,43). Since breast tumors commonly overexpress ras p21, we investigated whether breast cancer patients exhibited increased blood levels of ras and whether these increased levels predicted case-control status. Using our sample bank from the present study, 94 blood samples were analyzed and 29% of the case samples and 12% and 13% respectively of the healthy and benign breast disease control samples were positive for ras p21. After controlling for age and ethnicity the presence of ras in blood significantly predicted case status (OR=3.8, p=0.04).

iii. Blood DDE Levels Among Latina Women in Northern Manhattan

A portion of our present breast cancer study sample is drawn from a large Latina population that immigrated to the United States from the Dominican Republic and settled in northern Manhattan. Many of these subjects recall intensive government programs of DDT application that included the spraying of homes and aerial spraying of agricultural areas. Since prior studies have found an association between blood levels of DDE (a metabolite of DDT) and breast cancer it was of interest to determine whether these intensive exposures have led to increased blood DDE levels in this portion of our study population. Under separate funding, blood plasma samples from 19 healthy controls, 15 benign breast disease controls and 14 cases were analyzed for DDE levels using gas chromatography with electron detection. DDE levels ranged from 0.2 - 113.2 ppm with a mean of 18.3 ppm. No significant differences could be detected between cases and controls, possibly due to the small number of subjects.

DDE levels in the healthy group were compared to levels previously measured in 24 healthy non-Dominican women from the New York Metropolitan region who were treated at the Columbia-Presbyterian Medical Center for breast cysts. DDE levels were 3.22 times higher in the Dominican women than in the non-Dominican women (14.76 ppm vs. 4.56 ppm, p=.005). The increased body burden of DDE seen in Dominican women suggests that they would be a good model population in

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which to study the possible link between DDE and breast cancer.

e. Complementary Studies

Using other available funds we have analyzed stored white blood cell DNA from our breast cancer cases and controls for polymorphisms in genes that mediate the metabolism/detoxification of the environmental carcinogens under study (44,45,46,47,48,49,50). 120 subjects have been genotyped for the acetylator genotype (NAT2 slow vs. fast); 134 subjects have been genotyped for the glutathione transferase Mu polymorphism (GSTM1 deleted vs. present); and 124 subjects have been genotyped for the MSP1 polymorphism in the CYP1A1 gene (rare allele present vs. absent). These polymorphisms have previously been associated with increased carcinogen-DNA adduct levels and/or increased breast cancer risk in exposed individuals (51,52,50). Thus far, 63% of the subjects have the slow acetylator genotype, 6% have the rare CYP1A1 allele, and 43% are deleted at the GSTM1 locus. The completed genotype data set will be combined with our case-control and adduct data and will be used to analyze possible gene-environmental interactions in breast cancer development.

Additionally, since once a lab is running immunohistochemical analyses it requires very little incremental effort to analyze an additional marker by this technique, we have analyzed an additional 42 samples for erbB-2. 21% of the samples were found to strongly overexpress erbB-2, which is consistent with the literature (53,54,55). This marker will be used to further sub-characterize the cases into more etiological homogeneous groups which may be more closely associated with particular environmental exposures (56,57).

CONCLUSION

Progress during the first two years has been excellent, with enrollment ahead of schedule. In year three, given the flow of laboratory data from our collaborators, the focus of our work will shift towards analysis of these data. We anticipate that this study will provide important insights into the environmental etiology of breast cancer. Additionally, our strategy of banking tissue and blood samples will continue to provide a valuable resource for future projects.

Table 2.
Descriptive Analysis of Biomarkers with Continuous Data

Biomarker	Mean	SD
PAH-DNA adducts (MWBC)	6.99 per 10 ⁸ nucleotides	3.15 per 10 ⁸ nucleotides
HA-DNA adducts (MWBC)	Non-detect	NA
PAH-DNA adducts (Tissue)	0.44	0.11
DDE (Plasma)	18.3 ppm	25.2 ppm

Table 3.
Descriptive Analysis of Biomarkers with Categorical Data

Biomarker	Percent Positive
Smoking related-DNA adducts (MWBC)	13%
p53 (Tissue)	35%
cyclin D1 (Tissue)	27%
Ras p21 (Plasma)	18%, OR=3.8, p=0.04
NAT2 slow (WBC)	63%
GSTM1 deleted (WBC)	43%
CYP1A1 rare allele (WBC)	6%
erbB-2 (Tissue)	21%

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REPLY TO
ATTENTION OF:

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1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

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